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Evaluation of an enzymatic method for determining creatinine in plasma

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SUMMARY An enzymatic kit method for the determination of plasma creatinine was optimised for use with a centrifugal analyser and its performance characteristics and practicability compared with an end point and a kinetic Jaffé-based method. The enzymatic method exhibited several advantages over Jaffé-based methods—namely, smaller sample size, rapid sample throughput (200 per hour), and improved specificity. Glucose, acetoacetate, and cefoxitin did not interfere with the enzymatic method, although bilirubin did cause a negative interference which depended on both creatinine and bilirubin concentrations. The enzymatic method has particular clinical application in neonates, diabetic ketotic patients, and those receiving cephalosporins.

Routine clinical biochemistry laboratories use several methods for the estimation of plasma and urinary concentrations of creatinine, most of which are based on the Jaffé reaction. There are major analytical problems associated with the use of the Jaffé reaction, however, in particular those relating to positive and negative interference by chromogens. More than 50 chromogenic interferents have been documented.[1] Many modifications of the original Jaffé method have been developed to reduce interference by such substances in plasma, but these vary in their degree of success.

Enzymatic methods for creatinine determination have been developed in an attempt to overcome some of the problems inherent in the Jaffé-based methods. The underlying test principle of a new commercially available method entails a series of sequential enzyme-mediated steps which result in the formation of hydrogen peroxide. A Trinder indicator system is the final step in the reaction sequence and is responsible for the formation of an intense red colour with a maximum absorbance at a wavelength of 510 nm. The reaction sequence is summarised in table 1.

This method recently underwent a multicentre evaluation, in which 16 laboratories, using a variety of manual and mechanised methods and manufacturers' recommended protocols participated in the analytical evaluation.[2]

In this report, the enzymatic method was specifically optimised for use on the Cobas Bio centrifugal analyser (Roche Analytical Instruments, Nutley, New Jersey, USA) The analytical performance and practicability of the optimised method for routine use was assessed and compared with both a kinetic and end point Jaffé-based method. The clinical utility of the method was also examined.

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Material and methods

CREATININE METHODS

Enzymatic creatinine method

Enzymatic creatinine analyses were performed using the Creatinine PAP (CREA) enzymatic colorimetric test kit (Boehringer Mannheim, Mannheim, West Germany; catalogue No 836 885).

Preparation and stability of reagents

Reagent 1 (buffer/enzymes/4-aminophenazone) Potassium phosphate buffer (22 ml) (100 mmol/l, pH 7·9)

Table 1 *Reaction sequence of enzymatic method for assay of creatinine*

Creatinine + H_2O	creatininase →	Creatine
Creatine + H_2O	creatinase →	Sarcosine + urea
Sarcosine + H_2O + O_2	sarcosine oxidase →	Glycine + HCHO + H_2O_2
H_2O_2 + phenol derivative + 4-amino-phenazone	peroxidase →	Red benzoquinone-imine dye

were added to the powdered contents of phial 1, which contained creatinase, sarcosine oxidase, peroxidase and 4-aminophenazone. The resulting solution had the following composition: potassium phosphate buffer 100 mmol/l, pH 7·9; potassium hexacyanoferrate II 5 μmol/l; 2,4,6-tribromo-3-hydroxybenzoic acid 8·6 mmol/l; detergent 0·3%; 4-aminophenazone 0·8 mmol/l; creatinase >20 U/ml; sarcosine oxidase >12 U/ml; peroxidase >1 U/ml; ascorbate oxidase >10 U/ml. This reagent is stable for two weeks at 4° to 12°C and for two days at 15° to 25°C. It must be stored protected from light.

Reagent 2 (starter reagent, buffer/creatininase) Three reagent tablets were placed into the phial of buffer provided (phial 2) and allowed to dissolve. The resulting solution contained: potassium phosphate buffer 20 mmol/l pH 7·9; detergent 0·3%; creatininase >164 U/ml. This solution is stable for two weeks at 4° to 12°C and for two days at 15° to 25°C.

COMPARATIVE JAFFÉ-BASED METHODS

End point Jaffe (with dialysis)

Creatinine analyses were also performed on a Technicon SMA 6/60 Autoanalyser (Technicon Instruments Corporation, Tarrytown, New York 10591, USA) by continuous flow analysis using an end point Jaffé-based method which incorporated a dialysis step. Colour produced was measured at a wavelength of 520 nm.

Kinetic Jaffe

Creatinine concentrations were also determined using the Beckman Astra 8 (Beckman Instruments Incorporated, Clinical Instruments Division, Brea, California 92621, USA). Colour production was measured at a wavelength of 520 nm 25·6 seconds after the sample had been introduced into the Jaffé reagent.

The quality control materials used in this study were Autonorm (Nycomed AS, Oslo, Norway) and Wellcontrol Abnormal Unassayed (Wellcome Diagnostics, Dartford, England. DA1 5AH).

Technicon Diagnostics Set Point SMA Calibrator (Technicon Instruments Co. Tarrytown, NY. 10591) and Beckman Astra Aqueous Calibrator (Beckman Instruments Inc) were used as standards for the evaluation. The creatinine content of both solutions was 440 μmol/l.

INTERFERING SUBSTANCES

Patient pools

A series of three patient plasma pools of low, moderate, and high creatinine concentrations (87, 332, and 572 μmol/l, respectively) were prepared. The stated concentrations of analyte in each pool represented the mean value following triplicate analysis of each pool on the Technicon SMA 6/60 Autoanalyser. To each pool, known concentrations of the following substances were added.

Glucose (5 mg and 10 mg) (Ajax Chemical Company, Sydney) were weighed into 2 ml aliquots of each of the three patient pools to give final concentrations of 27·5 and 55 mmol/l of glucose, respectively, in each sample. The two values were consistent with those observed in uncontrolled diabetes mellitus.

Acetoacetate a stock solution (acetoacetate, lithium salt, Sigma Chemical Company, St Louis, Missouri 63178, USA) with a concentration of 54 g/l was prepared. Stock solution (5 μl and 10 μl) were added to 1 ml aliquots of each of the three patient pools to give final concentrations of 2·5 and 5·0 mmol/l of acetoacetate, respectively. These concentrations approximated those readily attained in the plasma of patients with diabetic ketoacidosis.

Bilirubin A stock solution (bilirubin, crystalline, from bovine gallstones, Sigma Chemical Company, St Louis, Missouri USA) of 10 mmol/l was prepared as follows: 117 mg of bilibrubin was dissolved in a solution of 2 ml of 0·1 M sodium carbonate (Ajax Chemical Co) and 1·5 ml of 0·1 M sodium hydroxide (Ajax). Once dissolved, this solution was made up to a volume of 20 ml with pooled plasma. This stock solution (10, 20 and 30 μl) was then added to 1 ml aliquots of each of the three patient pools. Final bilirubin concentrations was measured on a Unistat Bilirubinometer (Reichart-Jung) and gave values of 91, 179, and 267 μmol/l, respectively.

Cefoxitin a cephalosporin antibiotic (0·9 mg and 1·8 mg) (cefoxitin sodium, Merck, Sharp, and Dohme, Grandville, New South Wales) were added to 2 ml aliquots of each of the three patient pools to give final cefoxitin concentrations of 1 and 2 mmol/l, respectively. These concentrations were consistent with therapeutic concentrations for this antibiotic.

Results

OPTIMISATION OF THE REACTION CONDITIONS FOR THE ENZYMATIC METHOD

The centrifugal analyser settings recommended by the reagent kit manufacturer are listed in table 2 (column A). These variables specify that the total reaction period should extend over eight minutes. This time comprises an initial four minutes of preincubation of reagent with sample, followed by a further four minutes of reaction period after addition of the starting reagent containing the limiting enzyme creatininase. Reaction readings are obtained at only two points, one at the beginning and one at the end of the four minute reaction period.

The test variables recommended by the manufacturer were initially evaluated. The four minute preincubation period was found to be necessary, as the

Table 2 *Reaction conditions on Cobas Bio for measurement of creatinine*

Instrument settings		*A*	*B*
1	Units	μmol/l	μmol/l
2	Calculation factor	0	0
3	Standard 1 concentration	440	440
4	Standard 2 concentration	440	440
5	Standard 3 concentration	440	440
6	Limit	1600	1600
7	Temperature (°C)	37	37
8	Type of analysis	6	6
9	Wavelength (nm)	510	510
10	Sample volume (μl)	7	7
11	Diluent volume (μl)	30	30
12	Reagent volume (μl)	200	200
13	Incubation time (s)	240	240
14	Start reagent volume (μl)	40	40
15	Time of first reading (s)	10	60
16	Time interval (s)	240	10
17	Number of readings	2	9
18	Blanking mode	1	1
19	Printout mode	1	1

reaction was non-linear during the first 240 seconds after addition of start reagent. Using patient material with a high creatinine concentration (1300–1600 μmol/l), instrument settings were further investigated.

Linearity with samples containing high creatinine concentrations was observed for only 150 seconds after the addition of starting reagent. The time interval during which the reaction was monitored was therefore shortened to exclude the non-linear portion of the reaction. Concurrently, the reaction was monitored at more frequent (10 second) intervals as opposed to the 20 second intervals used earlier.

Closer monitoring of the reaction kinetics using these modified variables showed non-linear kinetics during the first 50 seconds following addition of starter, with linearity observed only during the 50 second to 150 second period of the reaction.

Variables were therefore revised again to confine the period of reaction monitoring to between 60 and 150 seconds following incubation, with a total of nine readings being made at 10 second intervals. A series of patient samples with creatinine values ranging from 85 to 1460 μmol/l were run according to these new variables. Reaction kinetics were shown to be linear for all samples throughout the period of monitoring.

The series of modifications described allowed the linear range of the method to be extended (to 1600 μmol/l), the reaction monitored at shorter reading intervals, and the total reaction time reduced by 90 seconds from eight minutes to six and a half minutes, with no difference in absolute creatinine concentration being observed with the shorter reaction time. The final optimised reaction conditions for the enzymatic creatinine method are listed in table 2 (column B).

PRECISION STUDIES

Intrarun and interrun precision data were obtained using five different samples, comprising three patient plasma pools containing low, moderate, and high concentrations of creatinine together with the two quality control materials routinely used in the laboratory of the authors (Autonorm and Wellcomtrol). For interrun precision studies, aliquots of the three patient pools were frozen at 0°C. The results obtained for intrarun and interrun precision studies are summarised in table 3.

Coefficients of variation reported for patient samples were smaller than those reported for the quality control materials. This may be due to a combination of the different matrix of the quality control materials (lyophilised, and of bovine origin) and the contribution of operator error to imprecision, as quality control materials were reconstituted daily from lyophilised powder, often by different technicians.

ACCURACY

The accuracy of the optimised enzymatic method was determined by analysing 100 hospital patient samples in duplicate. The resulting mean value for each of the duplicate pairs was compared with the value obtained on the same samples using the Jaffé end point method on the Technicon SMA 6/60 and the kinetic Jaffé method on the Beckman Astra 8.

The results were assessed using linear regression and indicated that the creatinine concentrations reported using the enzymatic technique were not significantly different to those reported using Jaffé-based methods: enzymatic μmol/l (y) = 0·99 (end point Jaffé) ×

Table 3 *Results of precision studies for optimised creatinine assay*

	Low	*Moderate*	*High*	*Autonorm*	*Wellcontrol*
Intrarun (n = 10):					
$\bar{x}$ (μmol/l)	125	375	722	84	402
SD (μmol/l)	2·6	7·5	13·9	4·8	4·4
CV (%)	2·1	2·0	1·9	5·7	1·1
Interrun (n = 10):					
$\bar{x}$ (μmol/l)	123	386	741	78	410
SD (μmol/l)	8·0	18·0	21·0	8·0	10·0
CV (%)	7·0	4·5	3·0	10·5	2·5

Plasma with low, moderate, and high creatinine was evaluated, in addition to the two quality control materials.

Table 4 *Summary of effects on common interfering substances on enzymatic and kinetic Jaffé methods for plasma creatinine*

Interferent	*Final concentration of interferent*	*Enzymatic (μmol/l)*			*Kinetic Jaffé (μmol/l)*		
		Low	*Moderate*	*High*	*Low*	*Moderate*	*High*
Glucose (mmol/l)	0	86	338	595	81	311	545
	27·5	85 (−1)*	340 (+1)	600 (+1)	93 (+16)	319 (+3)	553 (+2)
	55	87 (+1)	344 (+2)	594 (0)	96 (+19)	325 (+5)	565 (+4)
Acetoacetate (mmol/l)	0	85	339	601	81	311	545
	2·5	82 (−3)	328 (−3)	592 (−1)	142 (+75)	375 (+21)	619 (+13)
	5	79 (−7)	330 (−3)	583 (−3)	198 (+144)	445 (+43)	680 (+25)
Bilirubin (μmol/l)	0	87	332	572	81	317	551
	100	76 (−13)	291 (−12)	509 (−11)	64 (−22)	297 (−6)	535 (−3)
	200	60 (−31)	253 (−24)	456 (−20)	68 (−6)	290 (−9)	526 (−5)
	300	47 (−46)	218 (−34)	396 (−31)	65 (−20)	282 (−11)	505 (−8)
Cefoxitin (mmol/l)	0	83	333	583	89	312	544
	1	83 (0)	333 (0)	582 (0)	269 (+202)	446 (+43)	711 (+31)
	2	82 (0)	328 (−2)	576 (−1)	349 (+292)	598 (+92)	863 (+59)

*Figures in parentheses represent percentage difference in creatinine following addition of interferents.

−0·99 μmol/l; r^2 = 0·99 and enzymatic μmol/l (y) = 1·02 (kinetic Jaffé) × −2·58 μmol/l; r^2 = 0·99).

LINEARITY

Linearity was confirmed up to a concentration of 1600 μmol/l by serial dilution of a patient sample containing a high creatinine concentration.

REFERENCE RANGE

Reference values were determined by analysing plasma samples from 300 presumably healthy blood donors over a period of days in batches of 50. Reference ranges were then calculated using the Hoffman plot technique.[3] For adults, 55–122 μmol/l (n = 300); for men, 70–123 μmol/l (n = 150); for women, 49–108 μmol/l (n = 150). This reference range was comparable with that currently used in the authors' laboratory (60–120 μmol/l) derived using the end point Jaffé method. The difference in the values obtained for the male and female subpopulations can be atrributed to sex differences in muscle mass.

INTERFERING SUBSTANCES

Commonly encountered interfering substances of the Jaffé-based methods include glucose, acetoacetate, bilirubin, and cefoxitin.[4] Glucose and bilirubin both inhibit the reaction between creatinine and alkaline picrate. Glucose slowly reduces picric acid to picramate,[5] while bilirubin, under alkaline conditions, is oxidised to biliverdin, causing a decrease in absorbance at 520 nm.[6] Acetoacetate and cefoxitin, conversely, react directly with alkaline picrate. Acetoacetate, in fact, reacts more rapidly with picrate than does creatinine.[7] The thiopen nucleus is the active moiety in the cefoxitin molecule which reacts with the Jaffé reagent.[8] The effects of these known interferents on the enzymatic method were investigated and compared with their effect on the kinetic Jaffé method used on the Beckman Astra 8. Analyses were performed at least in duplicate. Results for all interfering agents are summarised in table 4.

The results for glucose indicated that it does not interfere with the enzymatic test method. A small degree of positive interference (+19%) was observed at the lowest creatinine concentration with the kinetic Jaffé method, but higher concentrations of creatinine were unaffected by the two levels of glucose tested. The results for acetoacetate showed that while the enzymatic method was unaffected by the addition of acetoacetate up to concentrations of 5 mmol/l, there was a considerable positive increase in the apparent creatinine concentration with the kinetic Jaffé method. This increase in apparent creatinine concentration was both directly proportional to the acetoacetate concentration and inversely related to the creatinine concentration—that is, the positive effect being highest at the lowest concentration of creatinine.

Our results indicate that the kinetic Jaffé method is not affected by the addition of bilirubin, but with the enzymatic method there is a significant depression in the actual creatinine concentration following the addition of bilirubin. The magnitude of the decrease depends entirely on creatinine concentration and bilirubin concentrations. Bilirubin interference in peroxidase-coupled enzymatic assays entailing the spectrophotometric measurement of hydrogen peroxide has been reported previously.[9–11] The mechanism by which bilirubin interference occurs has been explained by a combination of both spectral and chemical effects.[11] Spectral interference results from overlap of the broad 460 nm absorption band of bilirubin with the absorption band of the Trinder chromophore. Chemical interference is postulated to be due to a peroxidase reaction intermediate reacting with bilirubin, thereby decreasing the final amount of chomophore formed. The latter interference can be

eliminated by the addition of ferrocyanide to the reaction mixture, which stabilises the reaction intermediate.[12] Ferrocyanide is present in the reaction mixture of the reagent kit under evaluation, but only at a final concentration of 5 μmol/l. This concentration may be too low to effect adequate stabilisation.

The addition of the cephalosporin antibiotic cefoxitin resulted in a significant positive increase in creatinine concentrations with the kinetic Jaffé method. The positive interference observed was greatest at the lowest concentration of creatinine. No such interference was recorded when the enzymatic method was assessed.

CLINICAL UTILITY OF THE ENZYMATIC METHOD

The advantages in specificity of the enzymatic method were also shown in the following two clinical cases.

Case 1

A 16 year old female, an insulin dependent diabetic, presented to casualty in a confused state. She was volume depleted and pre-renal failure was suspected. The results of initial biochemical investigations are shown in table 5. The creatinine concentration of 235 μmol/l was assayed by the routine Beckman Astra. To assist the clinical decision on appropriate fluid management the specimen was reanalysed using the enzymatic method; a value of 81 μmol/l was found, suggesting normal renal function. The high concentration of acetoacetate in the patient's plasma had artefactually raised the apparent creatinine concentration to 235 μmol/l when assayed by the kinetic Jaffé method.

Case 2

An 18 month old infant presented with a three day history of persistent rhinorrhoea and modest anorexia, together with a 12 hour history of high fever, irritability, episodic drowsiness and vomiting. Examination showed prominent neck stiffness and possibly slight dehydration. A presumptive diagnosis of meningitis was subsequently confirmed by examination of cerebrospinal fluid. The patient was given cephalosporin cefotaxime (500 mg intravenously) and intravenous fluids at half normal maintenance volume. Plasma electrolytes over the next 24 hours are shown in table 6.

The admission hyponatraemia was thought to be

Table 5 *Biochemistry results on 16 year old insulin dependent diabetic (A) on admission; (B) and (C) three and 24 hours after starting treatment*

	A	*B*	*C*		
	(0855 h)	*(1200 h)*	*(0900 h)*	*Units*	*Reference range*
Sodium	135	137	135	mmol/l	132–144
Potassium	5·3	4·7	3·7	mmol/l	3·0–4·7
Chloride	100	109	107	mmol/l	93–108
Bicarbonate	9	6	14	mmol/l	21–32
Urea	8·3	7·3	2·9	mmol/l	3·0–8·0
Creatinine					
Kinetic Jaffé	235	225	76	μmol/l	60–120
Enzymatic	81			μmol/l	55–122
Glucose	39·8	19·0	11·7	mmol/l	3·0–5·5
Lactate	3·1			mmol/l	<2·0
β Hydroxybutyrate	7·6			mmol/l	<0·3
Acetoacetate	3·1			mmol/l	<0·1
Anion gap	31	27	18	mmol/l	7–17

Table 6 *Plasma biochemistry results in 18 month old baby receiving cephalosporin for meningitis*

	A Day 1	*B Day 2*	*C Day 2*	*D Day 2*		
	(2100 h)	*(1115 h)*	*(1500 h)*	*(2315 h)*	*Units*	*Reference range*
Plasma						
Sodium	125	139	143	136	mmol/l	132–144
Potassium	4·6	4·4	5·3	3·8	mmol/l	3·0–4·7
Chloride	100	110	113	110	mmol/l	93–108
Bicarbonate	14	9	12	15	mmol/l	21–32
Urea	4·4	6·1	5·9	4·1	mmol/l	3·0–7·0
Creatinine						
Kinetic Jaffé	63	137	132	99	μmol/l	20–50
Enzymatic	NA	NA	28	23	μmol/l	

secondary to the syndrome of inappropriate antidiuretic hormone secretion, but the raised creatinine concentrations of 63, 137, 132 and 99 μmol/l suggested pre-renal failure. The biochemistry laboratory was consulted and enzymatic creatinine determinations were performed on the two latest plasma samples. The results were found to be normal for an infant of this age (table 6), which allowed intravenous fluid administration to be continued.

PRACTICABILITY

The practicability of the enzyme-based method was also assessed. The assay is simple to perform, readily adaptable to the emergency or on-call situation, utilises a very small sample size (7 μl) and is rapid, being capable of analysing 200 samples per hour. A minor disadvantage of the method is its relatively high cost.

Discussion

The enzymatic method for creatinine evaluated in this study showed considerable improvement on the specificity of the existing Jaffé-based methods, although a problem still exists with interference from bilirubin. The modified enzymatic method was both precise and accurate, used a small sample size, and was capable of a rapid throughput of patient samples. We conclude that the enzymatic method is suitable as a routine diagnostic laboratory method for the measurement of plasma creatinine, particularly for diabetic ketotic patients, neonates, and patients receiving cephalosporins.

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